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CHEMICALLY AND PHOTOPERIODICALLY INDUCED DIAPAUSE DEVELOPMENT IN THE EUROPEAN CORN BORER, OSTRINIA NUBILALIS[1]

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The term "diapause development" was coined by Andrewartha (1952) to designate the physiological changes requisite to the termination of the diapause state and the resumption of morphogenesis. The physiological processes constituting diapause development have never been positively identified.

The principal schemes that have been advanced to explain diapause and diapause development can be classified into two categories: (1) developmental inhibitor theories, and (2) biochemical defect theories. Under the first category are those explanations in which the insect's normal developmental patterns are postulated to have been temporarily suppressed by an accumulation of an inhibiting metabolite (Roubaud, 1922), a "diapause factor X" (Bodine, 1932), "diapause factors X and Y" (Salt, 1947), or a growth-inhibiting "diapause hormone" (Schneider, 1950; Hinton, 1953; Fukaya and Mitsuhashi, 1957; de Wilde and de Boer, 1961). Such hypotheses postulated that the insect's growth remained in an arrested state until the inhibitor had been eliminated. Diapause development would, therefore, be identified as the process of biochemical degradation or elimination of the growth inhibitor. This process was usually assumed to be accomplished more rapidly at low temperatures than at high temperatures, thus accounting for the frequent observation that diapause termination requires prolonged exposure to low temperatures (0° to 10° C.). The "developmental inhibitor" theories of diapause have proved to be of limited value, because they have generally proved to be inconsistent with the results of detailed experimental analysis (see Lees, 1955, for review).

"Biochemical defect" theories of diapause postulate that the normal morphogenic sequence is blocked, during diapause, by the absence of an essential substance —a metabolite, enzyme, or hormone. The currently accepted theory is of this type, and treats diapause as a growth hormone deficiency syndrome. This concept of

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diapause was developed largely through research on the pupal diapause of *Hyalophora cecropia* and other large saturniids (Williams, 1946, 1947, 1948, 1952). Diapause is thought to be caused by a failure (for unknown reasons) of the neurosecretory cells of the brain to provide the substances required for brain hormone production. The absence of brain hormone results in inactive prothoracic glands and, consequently, no production of ecdysone. Without the latter hormone, growth and differentiation are arrested and metabolism is suppressed. By this theory, diapause development has been characterized as a renewal of brain activity (for unknown reasons) and the subsequent reestablishment of normal hormone production (Van der Kloot, 1955; Williams, 1956).

Some aspects of the "hormone deficiency" theory of diapause have been confirmed in other instances of pupal diapause (Highnam, 1958; Schoonhoven, 1962; Ichikawa *et al.*, 1956), larval diapause (Church, 1955; Fukaya and Mitsuhashi, 1957), and adult diapause (de Wilde and de Boer, 1961). It is not at all certain, however, that all instances of diapause involve a shut-down of brain neurosecretion as the specific limiting biochemical defect (Van der Kloot, 1960; de Wilde, 1961; Cloutier *et al.*, 1962); in some cases other components of the endocrine system may be defective.

The present study is a product of a long-term research program on the physiological nature of diapause development and the role of periodism in the control of growth phenomena. The experimental insect employed, *Ostrinia nubilalis*, displays a facultative larval diapause that is induced primarily by short-day photoperiods (about 12-hour scotophases) (Beck and Hanec, 1960; Beck, 1962). The temperature range required for diapause development is nearly identical to that required for morphogenesis (McLeod and Beck, 1963). Diapause development is strongly influenced by photoperiod, and diapause can be reinforced in mature larvae through an appropriate manipulation of photoperiod (McLeod and Beck, 1963). These characteristics make the species an excellent experimental form for detailed study of diapause development.

Materials and Methods

The methods for rearing the European corn borer and for inducing diapause by means of short-day photoperiods (12 hours of light and 12 hours of dark per day) have been presented in detail in previous papers (Beck, 1962; McLeod and Beck, 1963), and need not be reiterated here.

Borer larvae in diapause were removed from the rearing medium at 22 days of age. They were then placed individually in shell vials, each of which contained a strip of moistened absorbent paper. The larvae were then used experimentally.

In studying the effect of photoperiod on diapause development, a technique was employed that was based on the experimental results of McLeod and Beck (1963). This method involved exposure of the diapause larvae to long-day photoperiods (16 hours of light and 8 hours of dark per day) for a period of 10 days. This treatment resulted in near-completion of diapause development. Larvae so treated were then placed under experimental conditions (short-day or continuous dark) for an additional 10 days, following which they were returned to long-day conditions and observed for pupation. Pupation is a post-diapause event, and occurs well after the completion of diapause development. In the absence of

a well-defined criterion marking the completion of diapause development, pupation was taken as the measured endpoint.

Chemical treatments were by injection into the abdominal hemocoel, employing a 30-gauge hypodermic needle mounted on a microinjector. A standard volume of 5 microliters was used in all cases. The chemicals administered were dissolved in water at concentrations calculated to provide the desired dosage in 5 microliters.

Results and Discussion

Photoperiod and diapause development. Diapause borer larvae, 22 days of age, were subjected to different photoperiodic schedules and were then observed for pupation. The results (Table I) demonstrate some of the effects of photoperiod on the rate of diapause development. Of those larvae that were subjected to long-day conditions (Table I, Schedule A), 50% had pupated by the 31st day.

Table I

Effect of different photoperiodic treatments on diapause development in the European corn borer (all larvae were reared under a short-day photoperiod and were 22 days of age at the beginning of the experiment)

Schedule	Photoperiodic treatment		Post-treatment photoperiod	Days to 50% pupation*	
	1st 10 days	2nd 10 days		Avg.	Range
A	LD**	LD	LD	31	29–34
B	LD	LD	DD***	27	25–32
C	LD	DD	LD	29	26–34
D	LD	DD	DD	30	26–34
E	LD	SD†	LD	42	37–44
F	LD	SD	DD	>50	—
G	LD	SD	SD	>50	—

* Number of days from beginning of experiment until 50% of the experimental group had pupated. The range values are the extremes from experimental replicates.

** LD = Long day (16-hour photophase, 8-hour scotophase).

*** DD = Continuous darkness.

† SD = Short day (12-hour photophase, 12-hour scotophase).

This developmental rate was not significantly changed by transferring the larvae to continuous darkness after 20 days of long-day exposure (Schedule B). The developmental rate established during the 20 days of long-day photoperiods was apparently unaffected by the dark treatment. The results from Schedules C and D also show about the same developmental rates, indicating that the rate was established during the first 10 days of treatment and was unaltered by subsequent periods of continuous darkness.

Under Schedule E of Table I, borers were exposed to long-day photoperiods for 10 days, were returned to short-day conditions for 10 days, and were then held under long-day conditions for pupation. These larvae reached the 50% pupation point at 42 days, a delay of about 10 days in comparison to the pupation rate among larvae on the previously discussed schedules. The 10-day delay in diapause development corresponded to the 10 days that the larvae spent under a

short-day photoperiod. This response shows two points of interest: (1) exposure to short-day photoperiods during the second 10 days of the treatment sharply reduced the rate of diapause development, and (2) diapause development is accumulative, because the effect of the first 10 days under long-day photoperiods was not reversed or lost as a result of the later short-day treatment. If, however, the short-day treatment was followed by holding conditions of continuous darkness (Schedule F), or continued short days (Schedule G), the low rate of diapause development was maintained. Very few of the larvae (from 10% to 25%) under Schedules F and G had pupated by the end of the experimental time of 50 days. The low developmental rate established in response to short-day photoperiods was maintained under conditions of continuous darkness just as it was under continued exposure to short-day photoperiods. The role of photoperiods in diapause development appears to be that of determining the developmental rate.

The results from schedules B, C and D, contrasted with those from Schedule F, suggest that continuous darkness is photoperiodically neutral; that is, it is a *status quo* condition under which the developmental rate established by previous photoperiods is maintained. Since it has been previously shown (Beck and Apple, 1961) that exposure to continuous light promotes a rapid diapause development, it is apparent that continuous darkness and continuous light exert quite different effects on diapause development.

Only a small percentage (25% to 34%) of diapausing corn borer larvae that were held indefinitely from 22 days of age under either darkness or short-day photoperiods eventually pupated. Experimental measurements of diapause developmental rates under long-day and short-day conditions have indicated that one day of continuous light or long-day photoperiod (16 hours of light per day) resulted in an increment of diapause development equivalent to that requiring approximately 5 days of a short-day photoperiod (12 hours of light per day).

The above results are interpreted as showing that diapause in the European corn borer is not a state of *arrested* development. It is, instead, a physiological state in which the rate of developmental processes has been much reduced; the low developmental rate results in a greatly prolonged fifth larval instar (the diapause stadium) and in low oxygen consumption and other biochemical characteristics that have been found to be associated with the diapause state. Although we have dealt only with the European corn borer, it seems likely that diapause in other species may similarly involve varying degrees of developmental rate reduction rather than outright arrest; if so, the term "diapause development," as proposed by Andrewartha, reflects a legitimate concept, and is not "paradoxical," as labeled by Harvey (1962).

Other workers have also found some photoperiodic effects on form determination and diapause induction to be accumulative, with long-day effects not being completely reversed by subsequent short-day photoperiods (de Wilde, 1958; Beck and Hanec, 1960; Müller, 1962; Norris, 1962; Adkisson *et al.*, 1963; Barker *et al.*, 1963; Lees, 1963). Obviously the characteristic is general, and not peculiar to either the European corn borer or the phenomenon of diapause development.

Chemical treatment and diapause development. A study was made to determine whether or not diapause development could be accelerated by the administration of different chemicals. It was hoped that some clues to the identity of biochemical processes involved in diapause development might thus be obtained.

Water balance has frequently been implicated in insect diapause and neurosecretion (Slifer, 1946; Koidsumi, 1952; Bucklin, 1953; Nayar, 1960), and the water content of diapausing corn borer larvae has been shown to be lower than that of nondiapause larvae (Beck and Hanec, 1960). The European corn borer is also known to require contact moisture before postdiapause morphogenesis can occur (Babcock, 1924). Diapausing borer larvae were treated with different amounts of water at different ages and by several different means of administration, including injection into the hemocoel, direct introduction into the foregut and hindgut, and direct introduction into the tracheal system. In no case was diapause development accelerated by water, although it was found that the larvae could tolerate about 7 microliters of distilled water injected into the hemocoel.

A number of pharmaceutical agents, known to be central nervous system stimulants of mammals, were tested at several concentrations. It was hoped that one or more of the stimulants would promote brain hormone production (or release), thereby accelerating diapause development. The substances administered to 22-day-old diapause borers included amphetamine phosphate, methyl phenidate hydrochloride, pipradrol hydrochloride, imipramine hydrochloride, pentylenetetrazol, nethamine, and ephedrine sulfate. Water solutions were injected, and the treated larvae were held under either continuous darkness or a long-day photoperiod. All results were negative; no acceleration of diapause development was observed.

Hogan (1961, 1962) reported that urea and certain other ammonium compounds would terminate the embryonic diapause of a cricket, *Acheta commodus* (Walk.). This finding was tested with the European corn borer by injecting groups of diapausing larvae with different amounts of ammonium acetate. The treated larvae were then held in continuous darkness and observed for pupation. Ammonium acetate had a pronounced stimulating effect on the rate of diapause development (Table II). The greatest response (% pupating) was obtained with a dosage of from 600 to 800 μg. per larva. This was a massive dosage, as mature

TABLE II

Effect of different amounts of ammonium acetate on the rate of diapause development in the European corn borer. (40 larvae per treatment; all treatments were by injection in 5 μl. water)

Ammonium acetate dosage (μg.)	Response after 45 days in continuous darkness		
	Mortality (%)	Survival	
		Pupae (%)	Larvae (%)
0 (control)	15	0	85
200	40	0	60
300	80	8	12
400	40	20	40
500	58	28	15
600	58	40	2
800	58	40	2
1000	63	32	5
5000	100	0	0

TABLE III

Effects of different ammonium compounds on the rate of diapause development in the European corn borer. (All treatments were by injection in 5 μl. water; 40 larvae per treatment)

Compound	Dosage (μg.)	Response after 45 days in continuous dark		
		Mortality (%)	Survival	
			Pupae (%)	Larvae (%)
Ammonium acetate	600	58	40	2
Ammonium carbonate	500	70	0	30
	600	43	30	27
	800	80	20	0
Urea	50	20	0	80
	100	60	20	20
	500	80	10	10
	800	50	0	50
Ammonium oxalate	100	40	10	50
	300	100	0	0
Ammonium tartrate	100	70	0	30
	500	85	0	15
	800	100	0	0

borer larvae average only 100 mg. in body weight. That the response was to the ammonium ion rather than to the anion was demonstrated by the effects of several other ammonium compounds (Table III). These results demonstrate an important point of similarity between larval diapause of the European corn borer and embryonic diapause in a cricket.

Although a number of the ammonium compounds stimulated diapause development and pupation, most were rather toxic at the high dosage levels required. In general, ammonium acetate was the least toxic and most efficient of the substances tested, and it was adopted as the standard for further research. Attempts were made to improve the efficiency of the system for administering ammonium acetate to the larvae. One method tried was to use an ammonium acetate solution to moisten the paper strips in the vials in which the larvae were held; no response was obtained. Ammonium acetate solutions were introduced into the lumen of the hindgut by the use of small plastic tubes. This route of administration was effective, but the operation was difficult and inexact because of leakage. Effective amounts of the chemical could also be introduced into the foregut, but again offered no operational advantages over injection into the hemocoel. The results of these experiments demonstrated only that the borer digestive system was capable of absorbing ammonium acetate while in diapause. In an effort to avoid high postoperative mortality, serial injections of small amounts over a period of several days were tried. The mortality and response data showed no significant improvement

over the administration of a single massive dose. The addition of different amounts of ammonium acetate to the larval rearing medium did not reduce the incidence of diapause among borer larvae reared under a short-day photoperiod.

A possible explanation for the ammonium effect on diapause development is that ammonium ions are required for the synthesis of other nitrogenous compounds, and a massive influx of ammonium forces the synthetic process to an effective nondiapause rate of production. According to this hypothesis, administration of the biochemical substrates actually involved in the synthesis would be effective at greatly reduced concentrations. In pursuing this hypothesis, we have tested a large number of amino and other nitrogenous compounds, but with uniformly negative results. Carbamyl phosphate, tryptamine, 5-hydroxytryptamine, several

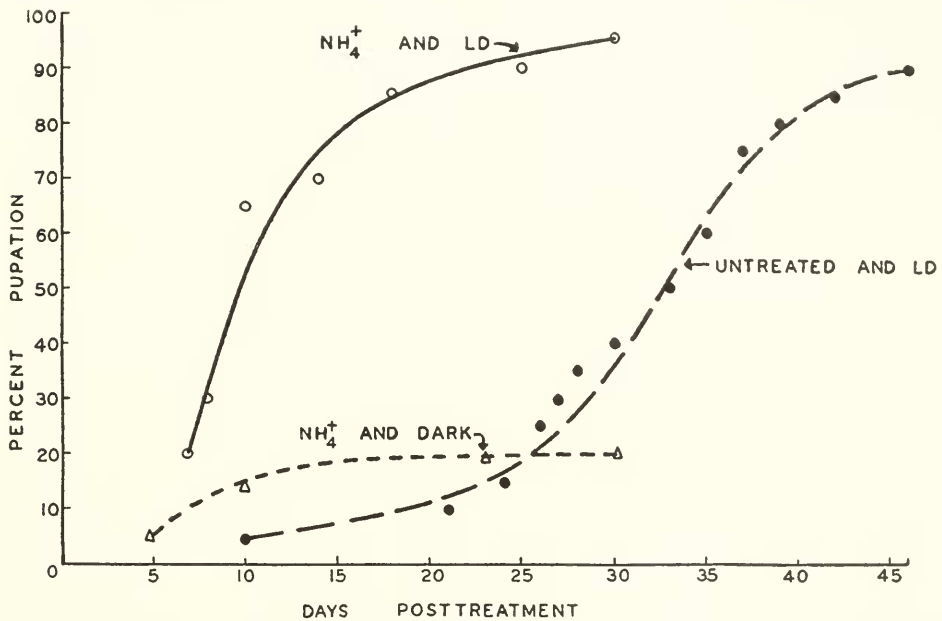

FIGURE 1. The combined effects of ammonium acetate (400 μg./insect) and photoperiod on diapause development in the European corn borer. (Untreated dark control record not plotted, because none pupated.)

amino acids, urea cycle intermediates, RNA synthesis intermediates, proline cycle intermediates, and several others have been tested. This aspect of the problem is under current investigation, but for the present we know the response only as the "ammonium ion effect."

Combined ammonium and photoperiodic effects. Ammonium ions accelerate diapause development, and long-day photoperiods exert the same effect. There may, therefore, be an interaction between the two accelerating factors. This hypothesis was tested in a series of experiments in which suboptimal doses of ammonium acetate were administered to diapause larvae, which were then held for pupation under either a long-day photoperiod or continuous darkness. The pupation records are shown in Figure 1. Diapause borers treated with 400 μg. of

ammonium acetate pupated at a high rate if held under a long-day photoperiod (50% pupating within 10 days), but at a low rate if held in the dark (only 20% within 30 days). Untreated control larvae reached the 50% pupation point in 32 days when under a long-day schedule, but none of the untreated dark controls pupated during the 46 days of the experiment.

When held under a long-day photoperiod, groups of larvae treated with 200 μg. of ammonium acetate per insect pupated at a rate no greater than that observed among borers not treated with ammonium ions. An intermediate response was obtained when larvae were treated with 300 μg. of ammonium acetate per insect.

Ammonium ions greatly accelerated diapause development, under post-treatment conditions of either darkness or a long-dark photoperiod. Ammonium ions did not eliminate the influence of photoperiod on diapause development, however. The factors that determine the rate difference in diapause development established by different photoperiodic schedules continued to operate in the presence of large quantities of ammonium acetate. On the basis of this evidence, it is suggested that ammonium ions are involved in biochemical processes constituting diapause development, but that some step in the utilization of ammonium ions is rate-limited through the action of photoperiod.

Summary and Conclusions

1. Diapause development in European corn borer larvae is rate-controlled by photoperiod. The rate of diapause development under conditions of continuous light or long-day photoperiods is approximately 5 times that occurring under a short-day photoperiod.

2. The rate of diapause development under conditions of continuous darkness depends upon the rate established by the photoperiods to which the larvae were exposed before being placed in the dark. Diapause larvae transferred from a short-day photoperiod into darkness continue to develop at the short-day rate. Conversely, diapause larvae exposed to about 10 days of long-day photoperiod continue to undergo diapause development at the long-day rate when transferred to dark conditions.

3. Although the rate of diapause development may be changed by changes in the photoperiod, diapause development is not reversible. Diapause development summates during the period of diapause.

4. Attempts to accelerate diapause development experimentally through increasing the water content of the larvae, or by the administration of chemical nervous system stimulants were uniformly unsuccessful.

5. Diapause development was experimentally accelerated by the administration of massive doses of ammonium acetate or other ammonium compounds. This finding indicates a probable similarity between larval and embryonic diapause development.

6. The ammonium ion effect did not obviate the role of photoperiod. The rate of diapause development was strongly influenced by photoperiod among larvae that had been treated with ammonium ions. The rate of diapause development observed in the presence of both ammonium ions and long-day photoperiods greatly surpassed the rates observed among larvae exposed to either factor alone.

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